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***Chlamydia trachomatis* hijacks intra-Golgi COG complex-dependent vesicle trafficking pathway**

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Abstract

Chlamydia spp. are obligate intracellular bacteria that replicate inside the host cell in a bacterial-modified-unique compartment called the inclusion. As other intracellular pathogens, chlamydiae exploit host membrane trafficking pathways to prevent lysosomal fusion and to acquire energy and nutrients essential for their survival and replication. The Conserved Oligomeric Golgi (COG) complex is a ubiquitously expressed membrane-associated protein complex that functions in a retrograde intra-Golgi trafficking through associations with coiled-coil tethers, SNAREs, Rabs and COPI proteins. Several COG complex-interacting proteins, including Rab1, Rab6, Rab14 and Syntaxin 6 are implicated in chlamydial development. In this study, we analyzed the recruitment of the COG complex and GS15-positive COG complex-dependent (CCD) vesicles to *Chlamydia trachomatis* inclusion and their participation in chlamydial growth. Immunofluorescent analysis revealed that both GFP-tagged and endogenous COG complex subunits associated with inclusions in a serovar-independent manner by 8 h post infection and were maintained throughout the entire developmental cycle. Golgi v-SNARE GS15 was associated with inclusions 24 h post infection, but was absent on the mid-cycle (8 h) inclusions, indicating that this Golgi SNARE is directed to inclusions after COG complex recruitment. Silencing of COG8 and GS15 by siRNA significantly decreased infectious yield of chlamydiae. Further, membranous structures likely derived from lysed bacteria were observed inside inclusions by electron microscopy, in cells depleted of COG8 or GS15. Our results showed that *C. trachomatis* hijacks the COG complex to re-direct the population of Golgi-derived retrograde vesicles to inclusions. These vesicles likely deliver nutrients that are required for bacterial development and replication.

Keywords

COG complex; GS15; Golgi; Chlamydia

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Conflict of interest statement

None declared.

Introduction

Chlamydiae are obligate intracellular Gram-negative bacteria associated with a multitude of human disease states. *Chlamydia trachomatis* is a major cause of pelvic inflammatory disease, ectopic pregnancy, and infertility among women; and also the leading cause of preventable blindness in the world (Schachter, 1989). Chlamydiae have a unique biphasic developmental cycle with 2 morphologically different forms, elementary body (EB) and reticular body (RB) (Rockey *et al.*, 2002; Scidmore *et al.*, 1996). Throughout their entire time in the host cell, chlamydiae remain within the confinements of the cytoplasmic membrane compartment termed inclusion, which very early during infection becomes fusogenic with a subset of exocytic vesicles originating from the Golgi region. Acquisition of sphingomyelin from Golgi is essential for chlamydial development; this acquisition is initiated very early in the cycle, a phenomenon driven by early chlamydial protein synthesis (Hackstadt *et al.*, 1996). While different chlamydiae exhibit differences in host tropism, spectrum of disease, and some aspects of basic biology, the nature of the inclusion is common within the genus and unique relative to all other intracellular pathogens (Rockey *et al.*, 2002). However, little is known about the mechanisms involved in the exit from endocytic and fusion with exocytic vesicle. Recent investigations in several labs identified a sub-population of Golgi-localized proteins, including CERT (Derre *et al.*, 2011), Rab1 (Rzomp *et al.*, 2003), Rab6 (Rejman Lipinski *et al.*, 2009), Rab14 (Capmany and Damiani, 2011) and SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) Syntaxin 6 (Moore *et al.*, 2011) which are specifically re-localized to chlamydial inclusions and are essential for bacterial survival and reproduction.

The Golgi apparatus is a hub for membrane trafficking pathways, organizing both anterograde and retrograde trafficking of molecules (for review see (Shorter and Warren, 2002)). It plays a key role in the intracellular trafficking, processing and secretion of glycoproteins and glycolipids (Warren and Malhotra, 1998). Studies in yeast and mammalian cells have led to the identification of several multi-subunit protein complexes that are thought to be involved in Golgi vesicle tethering and/or compartment function, including the COG (conserved oligomeric Golgi) (VanRheenen *et al.*, 1998; VanRheenen *et al.*, 1999), the TRAPP (Sacher *et al.*, 2001) and the GARP (Vps51-54) (Conibear and Stevens, 2000) complexes [for review see (Lupashin and Sztul, 2005)] The COG complex is preferentially localized to the *cis/medial* cisternae of the Golgi apparatus (Kim *et al.*, 1999; Spelbrink and Nothwehr, 1999; Ungar *et al.*, 2002), and is involved in Golgi membrane traffic (Bruinsma *et al.*, 2004; Ram *et al.*, 2002; Suvorova *et al.*, 2001; Suvorova *et al.*, 2002; Zolov and Lupashin, 2005). COG complex is a peripheral membrane hetero-oligomer which consists of eight subunits named COG1~8 (Fotso *et al.*, 2005; Kingsley *et al.*, 1986; Ram *et al.*, 2002; Suvorova *et al.*, 2001; Suvorova *et al.*, 2002; Ungar *et al.*, 2002; Whyte and Munro, 2001). Based on yeast genetic studies and observations with electron microscopy (Ungar *et al.*, 2002), the COG subunits have been grouped into two sub-complexes consisting of COG1 to COG4 for lobe A subcomplex and COG5 to COG8 for lobe B subcomplex. Mutations or depletions of the COG complex subunits lead to disruption of retrograde intra-Golgi trafficking of endogenous and exogenous proteins (Smith *et al.*, 2009; Zolov and Lupashin, 2005), improper glycosylation of glycoproteins and glycolipids (Bruinsma *et al.*, 2004; Shestakova *et al.*, 2006), and in humans causes multiple organ system pathologies referred to as congenital disorders of glycosylation (CDG) (Foulquier, 2009; Wu *et al.*, 2004). Intracellular accumulation of Golgi-derived transport vesicles that carry Golgi v-SNARE GS15/GOS15 is the earliest phenotype associated with the loss of COG complex subunits (Zolov and Lupashin, 2005). COG complex is orchestrating intra-Golgi vesicular trafficking through the functional interaction with coiled-coil tethers (Sohda *et al.*, SNAREs (Shestakova *et al.*, 2007; Suvorova *et al.*, 2002), Rabs (Smith *et al.*, 2009;

Sun *et al.*, 2007; Suvorova *et al.*, 2002; VanRheenen *et al.*, 1998) and COPI proteins (Ram *et al.*, 2002; Suvorova *et al.*, 2002; Zolov and Lupashin, 2005).

In cells infected with chlamydiae, Golgi resident protein golgin-84, one of COG-interacting coiled-coil golgins, is not re-localized to the inclusion, but instead is targeted for the specific protease cleavage (Heuer *et al.*, 2009; Rejman Lipinski *et al.*, 2009), while several other COG-interacting proteins, including Rab1, Rab6, Rab14 and Syntaxin6 are actively recruited to inclusion (Capmany and Damiani, 2011; Moore *et al.*, 2011; Rejman Lipinski *et al.*, 2009; Rzomp *et al.*, 2003), raising a question about possible involvement of the COG complex in chlamydial maturation.

Materials and Methods

Cell culture

HeLa cells (CCL-2; ATCC) were grown in Dulbecco modified Eagle's medium (DMEM/F-12) (HyClone) supplemented with 10% heat inactivated FBS (Atlas Biologicals) and containing penicillin 10 units/ml. Medium for stably expressing GFP-GS15 (N-terminal GFP tag), YFP-COG3 (N-terminal YFP tag) and COG8-GFP (C-terminal GFP tag) HeLa cells (this lab) was supplemented with 0.2 mg/ml G418 sulfate. Cells were transferred to antibiotic-free medium 24 h before infection.

Chlamydia trachomatis stock, infection and enumeration

Chlamydia trachomatis, serovar D or L2 strain and *Chlamydia muridarum* were propagated in *Mycoplasma*-free McCoy cells grown in DMEM media supplemented with 100 μ M nonessential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), 10% FBS, 50 mg/ml gentamicin sulfate, and 0.5 mg/ml cycloheximide. Infectious elementary bodies were isolated from McCoy cells by sonication 48 h post infection, washed in PBS, resuspended in SPG buffer (250 mM sucrose, 10 mM sodium phosphate, and 5 mM L-glutamic acid, pH 7.2), and stored in aliquots at -80°C . Stocks were titrated as described (Caldwell *et al.*, 1981). Infections of cells with *C. trachomatis* were carried as previously described (Prantner and Nagarajan, 2009). After chlamydiae were added at specific multiplicity of infection (MOI), cells were centrifuged at $1690 \times g$ at 37°C for 1 h. Infections were performed at 1 MOI, except when indicated otherwise. After the centrifugation, the media was aspirated and replaced with fresh media. Infected cells on coverslips were processed for immunofluorescence or inclusion forming units enumeration on a fresh McCoy monolayer at indicated times post infection (Caldwell *et al.*, 1981). Specifically cell lysates were prepared in 1.0 ml of SPG buffer, sonicated and centrifuged at $100 \times g$ to remove the debris. Lysate were overlaid on a McCoy titration plate (black bottom) at multiple dilutions (1:100, 1:1000 and 1:10,000) in a total volume of 200 μ l and infections were done by centrifugation as described earlier. Cells were fixed at 30 h and IFU were counted in 20 fields and calculated per ml. For all si-RNA knock down experiments, infections were performed 72 h after siRNA transfection.

DNA and siRNA transfection

Plasmid DNAs were isolated using the QIAprep Spin Miniprep Kit (Qiagen) and transfected into tissue culture cells with FuGene Transfection Reagent (Roche), according to the manufacturer's protocol. siRNA-duplexes for Rab6 (Sun *et al.*, 2007), COG3 (Zolov and Lupashin, 2005), COG8 (Smith *et al.*, 2009) and GS15 (Tai *et al.*, 2004) and siCONTROL1 (UAGCGACUAAACACAUCAA), were obtained from Dharmacon. Transfection was performed using Lipofectamine RNAiMAX siRNA Transfection Reagent (Invitrogen), following manufacturer's protocol. Knock down efficiency was confirmed by western blot and/or immunofluorescence.

Immunofluorescence microscopy

HeLa cells were grown on 12 mm glass coverslips (#1, 0.17 mm thickness) at 30% density one day before transfection. After knock down (KD) and infection with *C. trachomatis*, cells were fixed in 4% paraformaldehyde (16% stock solution; Electron Microscopy Sciences) and then treated with 1% Triton X-100 for one minute. Following incubation with 50 mM ammonium chloride for 5 min, cells were washed with PBS. For COG staining, the coverslips were subjected to freshly prepared 6M urea in PBS for 2 minutes, and washed with PBS. All coverslips were blocked twice for 10 min with 1% BSA, 0.1% saponin in PBS. Then, cells were incubated for 30–60 min at room temperature with primary antibody diluted in the 1% cold fish gelatin, 0.1% saponin in PBS (antibody buffer), washed four times with PBS and incubated for 30 min with fluorescently tagged secondary antibody (1:400 HiLyte Fluor; AnaSpec) in antibody buffer at room temperature. After that, coverslips were washed four times with PBS, rinsed 10 times with first PBS then ddH₂O, and mounted on glass microscope slides using Prolong® Gold antifade reagent along with DAPI (Invitrogen). Cells were imaged with the 63X oil 0.8 numerical aperture (NA) objective of a LSM510 Zeiss Laser inverted microscope outfitted with confocal optics. Image acquisition was controlled with LSM510 software (Release Version 4.0) SP1. All images presented are single z –plane sections.

SDS PAGE and western blots

Protein samples were lysed in hot 2% SDS and separated by 10% SDS-PAGE. Next, the gel was transferred onto 0.22 µm nitrocellulose membrane at 100V for 1 h. Membranes were blocked using LiCOR Odyssey Blocking Buffer for 20 minutes, incubated first with primary antibodies for 1 h, washed 4 times with PBS, and incubated with a secondary IgG antibody conjugated with IRDye 680 or IRDye 800 dyes. The blots were scanned and analyzed with an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

Antibodies

Antibodies used for immunofluorescent (IF) microscopy or western blotting (WB) were purchased through commercial sources, gifts from generous individual investigators, and/or generated by us via affinity purification. Antibodies (and their dilutions) were as follows: mouse monoclonal antibodies EVI H1 recognizing chlamydial LPS (IF 1:500), IncA (a gift from Dr. Daniel Rockey) (IF 1:50) and hCOG3 (this lab) (IF 1:1000), affinity-purified rabbit polyclonal antibodies hCOG3, hCOG6, hCOG8 (this lab) (WB 1:1,000 and IF 1:1,000), Giantin (Covance) (IF 1:3,000), GS15 (Synaptic Systems) (IF 1:300), Rab6 (Santa Cruz) (WB 1:400). IRDye 680 goat anti-rabbit, IRDye 800 goat anti-mouse for WB secondary antibodies were obtained from LI-COR Biosciences. Anti-rabbit HiLyte Fluor 488, anti-rabbit HiLyte Fluor 555, anti-mouse HiLyte Fluor 555, and anti-rabbit HiLyte Fluor 647 for IF were obtained from AnaSpec, Inc.

Transmission Electron Microscopy

Samples were treated according to Valdivia's lab protocol (Cocchiari *et al.*, 2008) with some modifications. In short, cells were fixed for 20 min on ice with 2.5% glutaraldehyde and 0.05% malachite green (EMS) in 0.1M sodium cacodylate buffer, pH 6.8. Samples were post-fixed for 30 min at room temperature with 0.5% osmium tetroxide and 0.8% potassium ferricyanide in 0.1 M sodium cacodylate, for 20 min on ice in 1% tannic acid, and for 1 h in 1% uranyl acetate at room temperature. Specimens were dehydrated with a graded ethanol series, and embedded in Araldite 502/Embed 812 resin (EMS). Ultrathin sections were imaged at 80 kV on FEI Technai G2 TF20 transmission electron microscope. Digital images were acquired with FEI Eagle 4kX USB Digital Camera.

Results

Co-localization of COG complex subunits with chlamydial inclusion

Chlamydial inclusion membrane acquires sphingomyelin and other nutrients from a host cell Golgi apparatus via a poorly defined mechanism (Moor et al, 2008), which is utilizing small GTPases Rab1, Rab6 (Rejman Lipinski *et al.*, 2009; Rzomp *et al.*, 2003), Rab14 (Capmany and Damiani, 2011) and trans-Golgi t-SNARE Syntaxin 6 (Moore *et al.*, 2011). We and others have shown previously that COG complex, a peripheral membrane protein complex, implicated in tethering of the retrograde intra-Golgi vesicles (Fotso *et al.*, 2005; Ungar *et al.*, 2002; Ungar *et al.*, 2006; Zolov and Lupashin, 2005), interacts with Rab1, Rab6 (Fukuda *et al.*, 2008; Sun *et al.*, 2007) and Syntaxin 6 (Laufman *et al.*, 2011). Given the central role of vesicle tethering factors in orchestrating vesicle trafficking, we examined whether GFP-tagged COG complex subunits co-localize with the chlamydial inclusion in *C. trachomatis* serovar L2-infected HeLa. Antibodies to chlamydial LPS and IncA were used to detect early (4 h, mostly peripheral), mid-cycle (8 h, both peripheral and clustered perinuclear) and late (24 h, large perinuclear) chlamydial inclusions. Both YFP-COG3 (Lobe A subunit) and COG8-GFP (Lobe B subunit) were localized to the Golgi apparatus in uninfected cells (Figure 1E,F) as observed previously (Shestakova *et al.*, 2007), and were not detected on early inclusion at 4 h (data not shown). YFP-COG3 (Figure 1A) and COG8-GFP (Figure 1B) specifically colocalized with LPS-positive mid-cycle chlamydial inclusions 8 h after infection indicating that both Lobe A and Lobe B sub-complexes of the COG complex are diverted to the inclusion membrane. Significant colocalization for COG complex subunits was also observed on or around the late (24 h) IncA-positive chlamydial inclusions (Figure 1C, D). In agreement with previous reports, Golgi trafficking factor Giantin, that is responsible for the tethering of one of the subclasses of intra-Golgi vesicles (Malsam *et al.*, 2005) did not localize to either mid-cycle or late chlamydial inclusions.

To confirm that the endogenous COG complex is being hijacked by chlamydial inclusion, localization of the endogenous COG complex subunits in infected cells was detected by indirect immunofluorescence (Figure 2). Indeed, almost all LPS-positive mid-cycle inclusions were co-labeled with anti-COG8 antibodies (Figure 2A) and significant COG8 signal was localized around the delimited membrane of IncA-positive large perinuclear inclusions (Figure 2B). Some of COG8-positive material was observed inside the inclusions. Importantly, recruitment of COG8 to chlamydial inclusion required active bacterial protein synthesis, since COG8 signal did not co-localize with LPS signal in chloramphenicol-treated cells (Figure 2C). These data verify antibody specificity and indicate that COG complex recruitment is facilitated by newly synthesized chlamydial proteins. Similar result was obtained with anti-COG3 and anti-COG6 staining (data not shown), supporting the hypothesis that the entire COG complex is partially mislocalized in Chlamydia-infected cells.

v-SNARE GS15 co-localize with late chlamydial inclusion

COG complex is one of the key regulators of the retrograde intra-Golgi trafficking. Research from our and other labs (Sohda *et al.*, 2010; Zolov and Lupashin, 2005) demonstrated that the acute down-regulation of COG complex function in HeLa cells disrupts intra-Golgi trafficking and causes accumulation of multiple transport vesicles that carry Golgi v-SNARE GS15. We tested if localization of this Golgi SNARE is affected in cells infected with *C. trachomatis* and found that both endogenous (Figure 3A–C) and GFP-tagged GS15 (Figure 3E) are specifically diverted from the Golgi and co-localized with the membrane of the late chlamydial inclusions. Surprisingly, we observed that a fraction of GS15 immunoreactive material was accumulated inside inclusions (Figure 3B, C) indicating that GS15-positive vesicles may not only fuse with the inclusion membrane, but also accumulate

within the inclusion. Again, as in the case of the COG complex, we found that the active bacterial protein synthesis is essential for the recruitment of GS15 to inclusion (Figure 3D).

COG complex and GS15 recruited to chlamydial inclusion in a serovar-independent manner

It has been shown previously that several host proteins, including the Rab6 effector Bicaudal D1 are associated with chlamydial inclusions in a serovar-specific manner (Moorhead *et al.*, 2007). To test if this is the case for COG complex and GS15, localization of the endogenous COG3, COG8 and GS15 was determined in HeLa cells infected with *C. trachomatis* serovar D. We observed that both COG subunits and GS15 were specifically co-localized with late chlamydial inclusions (Figure 4A, B), indicating that they are recruited to the inclusion membrane in serovar-independent manner by the mechanism that is likely to be different to one used by Bicaudal D1. Similar association of COG3 and GS15 with inclusion membrane was also found in HeLa cells infected with *C. muridarum* (Figure 4C).

GS15 is localized to chlamydial inclusions in a COG-dependent manner

After establishing that both Golgi trafficking factors, COG complex and GS15 are actively recruited to chlamydial inclusion membrane, we asked the question which molecule is recruited first and if their recruitment is dependent on each other. We and others have shown previously that Golgi localization and stability of GS15 is regulated by COG complex (Oka *et al.*, 2004; Zolov and Lupashin, 2005). We hypothesized that this scenario could be used in Chlamydia-infected cells. Indeed, in cells that stably express GFP-GS15, both COG8 (Figure 5A) and COG6 (Figure 5B) were recruited to LPS-positive small chlamydial inclusions 8 hours post-infection and these LPS/COG-positive structures did not possess any detectable GFP-GS15 signal. In contrast, both COG subunits and GFP-GS15 were co-localized with each other on the inclusion membrane 24 hours post-infection (Figure 5C, D, and Figure 6A)). Again, neither COG8 nor GFP-GS15 was localized to Chlamydia-positive membranes in cells treated with chloramphenicol (Figure 5E). Importantly in cells depleted for COG3 subunit of the COG complex, GFP-GS15 failed to localize on the inclusion membrane (Figure 6B), indicating that a functional COG complex is necessary for GS15 recruitment to chlamydial inclusions. Interestingly, in these cells COG6 signal was distributed diffusely in the cytoplasm without any concentration on the inclusion membrane, indicating that only a fully functioning COG complex is hijacked by chlamydial inclusion. Again, both COG6 and GFP-GS15 signals are co-localized with the inclusion membrane in control cells (Figure 6A). In cells depleted for GS15, the development of chlamydial inclusions was altered (see below), but these altered inclusions were still surrounded by COG6 and YFP-COG3 signals (Figure 6C), indicating that COG complex is recruited to inclusion in a GS15-independent manner.

COG complex and GS15 are essential for chlamydial development

COG complex subunits were associated with only some nascent peripheral inclusions, while GS15 was found only on late inclusions, suggesting that these molecules are not involved in the entry process. Indeed, the number of initial inclusions was not altered in cells downregulated for COG3, COG8 or GS15 (data not shown). To analyze the role of these molecules in chlamydial development, growth and replication, HeLa cells were transfected with COG8 siRNA, GS15 siRNA, control siRNA (negative control) or Rab6 siRNA (positive control) (Rejman Lipinski *et al.*, 2009) and infected with *C. trachomatis*, serovar D, 72h after siRNA transfection. At this point, downregulation of COG8, GS15 and Rab6 was ~90% complete, as assessed by western blot (Figure 7A) or fluorescent microscopy (data not shown). Localization of Golgi protein GM130 was largely unaltered in majority of siRNA treated cells (Figure 7B).

To address the effect of COG complex and GS15 depletion on bacterial growth and replication, infected HeLa cells were lysed at 48 h post-infection. The infectious EBs were titrated on a fresh McCoy monolayer by counting the Inclusions Forming Units (IFU) 30 h later. The amount of infectious bacteria recovered from COG8KD or GS15KD cells was significantly decreased compared to the control HeLa cells. The IFU assays demonstrated the negative impact of depletion of either COG complex or GS15 on chlamydial development (Figure 7C). Importantly, the extent of this negative effect was similar to the effect observed in Rab6 depleted cells.

The development of bacteria in control, COG8KD and GS15KD cells was further assessed by TEM microscopy. To better preserve membrane and lipid structures inside inclusions, fixation in the presence of malachite green (Cocchiario *et al.*, 2008) was employed. Absolute majority of visualized inclusions in COG8KD (92%, n=26) (Figure 8B) and GS15 (87%, n=16) (Figure 8C) cells demonstrated a presence of multiple membranous structures and aberrant (partially lysed) bacterial forms (Figures 8B and C, arrows). In contrast, similar membrane structures were observed with very low frequency (25%, n=20) in infected HeLa cells treated with control siRNA (Figure 8A). The effect on chlamydial inclusion content and yield of infectious particles implies the role for the COG complex and Golgi v-SNARE GS15 in intracellular development and growth of *Chlamydia* spp. in mammalian cells.

Discussion

In this study, we demonstrate that the peripheral membrane complex COG, the master regulator of retrograde vesicular trafficking in the Golgi apparatus, as well as v-SNARE GS15 are recruited to chlamydial inclusion in a temporal manner and this association is required for optimal chlamydial development. Work in our and other labs demonstrated the COG complex, physically and functionally interacts with the majority of Rab and SNARE proteins that are recruited to chlamydial membrane (Fukuda *et al.*, 2008; Laufman *et al.*, 2011; Smith *et al.*, 2009; Sun *et al.*, 2007), suggesting that this complex could be recruited to inclusions as well. Indeed, we found that COG complex appeared on the inclusion membrane as early as 8 hour post-infection, indicating that this recruitment may be one of the earliest points of communication between bacterial inclusion and intra-Golgi vesicular trafficking. A schematic model that depicts the recruitment of the COG complex and COG-interaction proteins to chlamydial inclusions is shown on Figure 9.

Chlamydia spp., like many other intracellular parasites have evolved diverse mechanisms to boost their survival and replication within mammalian cells (Hackstadt *et al.*, 1997). Interaction with the Golgi-derived vesicular pathway has been proposed as one potential mechanism enabling chlamydiae not only to escape from lysosomal fusion, but also to receive cholesterol and sphingomyelin from the host cell (Carabeo *et al.*, 2003; Moore *et al.*, 2008; Wolf and Hackstadt, 2001). Number of Golgi-derived host molecules, including Bicaudal D1 (Moorhead *et al.*, 2007), CERT (Derre *et al.*, 2011), Rab1 (Rzomp *et al.*, 2003), Rab6 (Rejman Lipinski *et al.*, 2009), Rab14 (Capmany and Damiani, 2011) and Syntaxin 6 (Moore *et al.*, 2011) are shown to be recruited to the chlamydial inclusion membrane, but the nature of this recruitment and the exact role of these molecules in chlamydial survival and replication is still unclear. COG complex is known to bind SNARE domain of Golgi SNARE proteins (Laufman *et al.*, 2011; Shestakova *et al.*, 2007). Further, at least three chlamydial Inc proteins, IncA, CT813 and CT233 showed similarities with SNARE motifs (Delevoe *et al.*, 2008). Therefore, one intriguing possibility is that one of these proteins is involved in hijacking of the COG complex to the nascent inclusion membrane. In support of this possibility, *C. trachomatis* inclusion membrane protein CT229 has been shown to interact with GTPase Rab4 (Rzomp *et al.*, 2006).

COG complex regulates trafficking of intra-Golgi vesicles that contain v-SNARE GS15. Furthermore, this SNARE protein is recruited to chlamydial inclusion later in development. Surprisingly, we observed that a fraction of GS15 and COG subunit immunoreactive material was accumulated inside inclusions indicating that COG complex and GS15-positive vesicles may not only fuse with the inclusion membrane, but also accumulate within the inclusions. Alternatively, intra-inclusion signal may indicate that COG subunits and GS15 aggregates are internalized by the inclusion. This intra-inclusion immunostaining is likely to be specific, since it was observed with several affinity-purified polyclonal (anti-GS15, anti-COG6, anti-COG8) and monoclonal (anti-COG3) antibodies, and these antibodies did not stain chlamydial inclusions that were blocked by chloroamphenicol. However, we could not exclude that some of this intra-inclusion staining could be due to cross-reactivity of antibodies used in this study with yet unidentified bacterial components at heavy bacterial loads 24 h post infection. Intriguingly, similar intra-inclusion accumulation of lipid droplets (Cocchiari *et al.*, 2008), sphingomyelin (Wolf and Hackstadt, 2001) and beta-catenin (Prozialeck *et al.*, 2002) were reported previously. The biological significance of sequestration of GS15 inside the inclusion is unclear. One possible speculation is that GS15 positive vesicles inside the inclusion facilitate nutrient transfer directly across the bacterial membrane. Interestingly, GS15 recruitment to inclusion is abrogated in COG deficient cells, indicating that functional COG complex is necessary for re-direction of trafficking of the GS15-bearing intra-Golgi vesicles toward bacterial inclusion. GFP-tagged GS15 and COG molecules were largely not present inside the inclusion, indicating that exogenously expressed (and often overexpressed) fluorescently-tagged proteins only partially recapitulate the behavior of the endogenous protein.

Consistent with the role for Golgi trafficking machinery in acquisition of nutrients by chlamydiae, recruitment of COG complex and GS15 to the chlamydial inclusion is essential for optimal chlamydial development. In cells depleted of COG complex and GS15, inclusions are filled with bacterial ghosts and other membranous remnants, while the yield of infectious bacteria is significantly diminished. This may be partially due to deficient trafficking of essential nutrients, including sphingomyelin and cholesterol, from the host Golgi, since the content of sphingomyelin is significantly reduced in COG deficient cells (Spessott *et al.*, 2010). One intriguing possibility is that COG8 KD and GS15 KD could induce a persistent state due to nutrient deprivation. However, knock down of both the COG complex or GS15 recruitment did not influence inclusion formation or inclusion numbers, suggesting that COG3, COG8, and GS15 are not essential for chlamydial entry and early events in escaping from the endocytic pathway. Conforming to this observation, no association of COG complex was observed at early time points (4 h) post infection (data not shown).

Previously, it has been shown that Rab6 is recruited to chlamydial inclusion in a species independent manner, interacting with all *C. trachomatis* strains (Rzomp *et al.*, 2003). However, in contrast to Rab6 recruitment, Rab6 effector BiCD1 interacts exclusively with *C. trachomatis* L2 and not with serovar B, serovar D, *C. muridarum* or *C. pneumoniae* (Moorhead *et al.*, 2007). Similar to Rab6, we observed that interaction of COG complex with chlamydial inclusion was observed with *C. trachomatis* D, L2 and *C. muridarum*, suggesting that these interactions are likely essential for all chlamydial strains. Furthermore, this would suggest involvement of a chlamydial Inc proteins conserved between *C. trachomatis* strains. However, further investigation is necessary using ocular *C. trachomatis* serovars, and other biovars such as *C. pneumonia* and *C. psittaci* strains, to determine the specificities of COG and GS15 interactions.

In summary, our work demonstrates an important role for COG complex and GS15 in chlamydial growth and development, right after the early inclusion is formed. The presence

of aberrant RB and membranous structures inside the inclusion in the absence of a functional COG complex and GS15, further indicates the important role of these vesicle transport factors in nutrient transport. It is likely that *C. trachomatis* is hijacking host COG complex subunits to the inclusion membrane via direct binding to its SNARE-like Inc proteins. The specific chlamydial protein interacting with COG complex is certainly of interest to determine in the future to understand the specific nature of this association.

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Abbreviations

COG	<i>conserved oligomeric Golgi</i>
EBs	<i>Elementary Bodies</i>
IFU	Inclusions Forming Units
KD	<i>knock-down</i>
RBs	Reticular Bodies
SNARE	<i>soluble N-ethylmaleimide-sensitive factor attachment protein receptor</i>

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Fig. 1. Recruitment of fluorescently tagged COG3 and COG8 to the chlamydial inclusions
 Stable YFP-COG3 (A,C) and COG8-GFP (B,D) HeLa cells infected with *Chlamydia trachomatis* L2 (MOI 1.0) were fixed either 8 h (A,B) or 24 h (C,D) after infection. Cells were stained for chlamydial LPS using conjugated mAbs LPS (HiLyte Fluor -555) for 8 h infection or IncA (HiLyte Fluor -555) for 24 h infection, Giantin (HiLyte Fluor-649), and DAPI. Control uninfected YFP-COG3 (E) and COG8-GFP (F) cells were stained for COG8, COG6, Giantin and DAPI as indicated. Slides were visualized using a Zeiss LSM 510 Meta Laser confocal microscope. Arrows indicate the membrane of chlamydial inclusions. The merge images are shown on the right. Scale bar, 10 μ m.

Fig. 2. Endogenous COG8 is associated with *C. trachomatis* inclusions

HeLa cells infected with *C. trachomatis* serovar L2 (A,B) or serovar D (C) (MOI 1.0) were fixed either 8 h (A) or 24 h (B, C) post-infection and analyzed by confocal microscopy. In (C) cells were treated with chloramphenicol 3 h post-infection to block bacterial protein synthesis. Cells were stained for chlamydial LPS (HiLyte Fluor-555) and COG8 (HiLyte Fluor-488) (A), GM130 (HiLyte Fluor-555), COG8 (HiLyte Fluor-488) and DAPI (B), or LPS (HiLyte Fluor-555), COG8 (HiLyte Fluor-488) and DAPI (C). The merge images are shown on the right. The intensity profiles showed COG8 (green) specifically localized in a close proximity to inclusion membrane surrounding actively growing bacteria (LPS (red) in

(A) and DAPI (blue) in (B)). Arrows indicate the membrane of chlamydial inclusions. Scale bar, 10 μ m.

Fig. 3. Colocalization of SNARE protein GS15 with *C. trachomatis*

HeLa cells (A–D) or HeLa cells stably transfected with GFP-GS15 (E) infected with *C. trachomatis* L2 (B,C,E) or D (A,D) for 24 h were fixed and labeled with antibodies and DAPI as indicated. In (D) cells were treated with chloramphenicol 3 h after infection to block bacterial protein synthesis. The merge images are shown on the right. In (C) four Z sections of merged GM130/GS15/DAPI staining are shown. Arrows indicate the membrane of chlamydial inclusions. Scale bar, 10 μm.

Fig. 4. Recruitment of GS15 and COG complex subunits to inclusions formed by *C. trachomatis* serovar D and *C. muridarum*

HeLa cells infected with *C. trachomatis* serovar D (A and B) or *C. muridarum* (C) for 24 h were fixed and labeled with antibodies against COG8 and GS15 (A), Giantin and COG3 (B), GS15 and COG3 (C) and DAPI. The merge images are shown on the right. Arrows indicate the membrane of chlamydial inclusions. Scale bar, 10 μ m.

Fig. 5. COG complex subunits localized to chlamydial inclusions before GS15

Stably transfected GFP-GS15 HeLa cells infected with *C. trachomatis* serovar L2 (A–C) or serovar D (D–E) were fixed either 8 h (A, B) or 24 h (C, D, E) after infection. In (E) cells were treated with chloramphenicol 3 h after infection to block bacterial protein synthesis. Cells were stained for chlamydial LPS (HiLyte Fluor-555) and COG8 (HiLyte Fluor-647) (A, E), DAPI and COG8 (HiLyte Fluor-647) (C), or chlamydial LPS (HiLyte Fluor-555) and COG6 (HiLyte Fluor-647) (B, D). The merge images are shown on the right. Arrows indicate the membrane of chlamydial inclusions. Scale bar, 10 μ m.

Fig. 6. Localization of GS15 to chlamydial inclusion is abrogated in COG3 KD cells

HeLa cells stably expressing GFP-GS15 (A, B), or YFP-COG3 (C) were treated with control siRNA (A), COG3 siRNA (B) or GS15 siRNA (C) for 72 h and then infected with *C. trachomatis* serovar D and fixed 24 h post infection. Cells were stained for COG6 (HiLyte Fluor-555) and for DAPI (A,B) or IncA (HiLyte Fluor-488) (C). The merge images are shown on the right. Arrows indicate the membrane of chlamydial inclusions. Scale bar, 10 μm.

Fig. 7. GS15, COG8 and Rab6 are important factors for *C. trachomatis* development

Western blot of control, GS15KD, COG8 KD and Rab6 KD HeLa lysates showing the efficiency of KD (A). COG8, GS15 and Rab6 were silenced by transfection of specific *siRNAs*. Cells were then fixed and stained with GM130 antibody and DAPI. Note that Golgi complex morphology has not been changed dramatically in knock-down cells (B). Down-regulation of COG8, Rab6 and GS15 affects infectious chlamydial yields significantly. HeLa cells were transfected with COG8siRNA, Rab6siRNA, GS15siRNA or control siRNA and infected 48h later with *C. trachomatis*, serovar D at 0.01 and 0.1 MOI. 48 h post infection, cells were harvested and infectious EBs obtained were determined by infecting a fresh McCoy cells layer and IFU determined. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-Way ANOVA and post-hoc Tukey multiple comparison between control and specific KDs (C).

Fig. 8. Ultrastructural analysis of chlamydial inclusions

HeLa cells were transfected with control siRNA (A), COG8 siRNA (B), or GS15 siRNA (C) and infected with *C. trachomatis* serovar D 72 h (MOI=5) after transfection. Cells were fixed 24 h after infection and processed for TEM. Representative inclusions (out of ~20 random sections) are shown at 6500 $\times$ magnification (Scale bar, 1 μ m) and at 25000 $\times$ magnification (Last image of each panel, Scale bar, 0.2 μ m). Note that inclusions in COG8 KD and GS15 KD cells contained large amount of membranous material and bacteria ghosts (arrows) in addition to reticular bodies (RB). At least some membranous material inside the inclusion in COG8KD cells is originated from the lysed RBs (D, arrowheads). Significantly more membranous material was observed in inclusions of COG8 KD (24/26 inclusions) and GS15 KD cells (14/16 inclusions) compared to control (5/20 inclusions). $P<0.001$ (control vs COG8 KD) and $P=0.002$ (control vs. GS15 KD) by Fisher exact test.

Fig. 9. Model for the recruitment of the COG complex and COG-interacting proteins to chlamydial inclusions

Solid arrows represent known protein-protein interactions. Dotted arrows represent recruitment to chlamydial inclusions. The model shows that recruitment of COG complex is essential for GS15 recruitment to the inclusion.